5657

REMOVAL OF COLLAGENS VI AND XII BY BEAMHOUSE CHEMISTRY

by
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Abstract

Hides are prepared in the beamhouse for tanning by removing nonfibrous proteins from among the collagen fibrils. We now know that some of this protein also contains types of collagen that do not form fibrils. This material might be expected to resist the drastic chemical treatments of the beamhouse, as does the collagen type I of the fibers. Using immunochemical stains, we have examined the persistence of two such nonfibrillar collagens, types VI and XII, through simulated liming, bating (trypsin digestion), and pickling. Both collagens persisted after treatment with lime, but the antigenic site on the molecule of the collagen type XII was removed by trypsin. Collagen type VI resisted all the treatments. Beamhouse treatments eliminate at least part of the type XII molecule, which is found on the surfaces of the fibrils, but leaves type VI, which is present in very fine strands that connect the fibrils of types I/III/V. These remnant proteins can complicate the use of syntans, fatliquors, and dyes, which act on the surfaces of the fibrils.

Introduction

Cattlehide is prepared in the beamhouse for tanning by removal of the hair, epidermis, and most of the interfibrillary macromolecules by soaking, liming, and perhaps pickling. During the past fifteen years the macromolecular components have been isolated and classified and consist of the following classes:

1. Fibril-forming collagen molecules
2. Fiber-associated collagen with interrupted triple helices
3. Molecules of the interfibrillary gel
4. Proteoglycans, mostly protein, attached tightly to fibrils
5. Adhesion molecules - glycoproteins like fibronectin.

Many members of each class have been isolated and biochemically analyzed for amino-acid sequence and molecular structure and function. Some have been genetically cloned. The information so obtained suggests that they may be important in beamhouse technology.

The first class above is plentiful in hide and, in the past, was what was understood to be "collagen." There are, however, other types of collagen in hide that are found only in small amounts and that cannot form fibrils. There are fourteen chemically different known types of collagen, conventionally referred to by Roman numerals that give the order in which they were discovered. The fiber-associated collagens with interrupted triple helices (FACIT) that have been found in hide are collagens type XII and XIV (collagen XII or collagen XIV)[1]. While their physiological properties are a matter of speculation, their presence or removal from the hide might influence the properties of the leather product since they are bound to the surfaces of the fibrils of the hide.

Further, the adhesive gel-like material found among the fibrils (No. 3 above) should be removed in the beamhouse. Most of the gel is removed, in order for tanning reagents to permeate and for the leather to dry to a soft consistency. The persistence of small amounts on surfaces of fibrils, however, could control the mechanical and dyeing properties of the final product. This material includes a minority collagen, type VI (collagen VI), which can bind to the collagen type I (collagen I) of the fibrils and to membranes of cells that are found on the fibrils before they are disrupted during curing and storage[3]. There is, therefore, a biochemical basis for the hypothesis that collagen VI, as well as proteoglycans and adhesion molecules, have an adhesive role.

While some proteoglycans are left in the leather from the beamhouse[4], there is no experimental confirmation of the removal or persistence of any of the minority collagens after hide processing.

The fibers of skin or hide are subdivided into dense fibrils about 100 nm in diameter. When stained with heavy metals, they can be seen in an electron microscope to have a banded structure, the bands corresponding to the horizontal alignment of polar parts of adjacent collagen molecules[5]. These fibrils are heterotypic, containing at least two types of fiber-forming collagens, types I and III and perhaps V[6]. Any of the three can form the fibrils alone or in combination with the others. Homotypic fibrils from types III or V, however, are thinner than those from type I, but otherwise look the same when stained and viewed in the electron microscope[7,8]. How a basic fibrillar structure comprising three types of collagen affects the properties of skin, hide, or leather has not been reported.

In this work we have investigated the removal of the FACIT collagen XII and the interfibrillary-gel collagen VI by simulated beamhouse treatments (liming, bating, and pickling) simulated on microscope slides. We have used immunological staining to evaluate the removal with a polyclonal antibody to bovine collagen VI and a monoclonal antibody to chicken collagen XII. Although we realize that a polyclonal antibody to bovine collagen XII might have given more straightforward results, as we explain later, we used the only antibody to a skin FACIT molecule that has been prepared and characterized at this time.

Materials and Methods

Type VI Collagen

Immunologic labelling for type VI collagen was performed on skin from six-month-old calf that had been frozen at −40°C. Frozen sections of calfskin (12 μm thickness) for light microscopy were collected on albumin-coated microscope slides, air-dried, and fixed with chilled acetone for 10 min. The sections were washed with phosphate-buffered saline (PBS) (130 mM NaCl, 7.6 mM Na_2HPO_4, 2.4 mM KH_2PO_4), pH 7.3 or 7.4, several times at 4°C.

To simulate liming, selected sections on the slides were treated with saturated calcium hydroxide for 16 hrs at 4°C and washed with cold PBS. To simulate sulfide unhairing, sections were treated with a solution containing 0.4% sodium sulfide, 1.0% sodium hydrosulfide and 1.6% lime for 6 hrs at 22°C and washed with cold PBS. To simulate bating, sections were treated with a 1 mg/ml solution of bovine pancreatic trypsin (Sigma chemical Co., #T 8642) for 2 hrs at 37°C and washed several times with cold PBS. These treatments were carried out singly and not in sequence. Control sections were placed directly into incubation with normal goat serum (NGS).

Following each treatment, sections were incubated with NGS for 15 min at 22°C in a moist chamber and rinsed with several changes of PBS at 4°C. The tissue was incubated with polyclonal rabbit anti-collagen VI (supplied by D.E. Engvall) for 1 hr at 22°C; background-blank sections were incubated only with PBS. This antibody, like anti-collagen XII, does not cross-react with collagen I or its gelatin, as will be evident. All sections were then rinsed several times with PBS. They were subsequently incubated with the secondary antibody, anti-rabbit IgG, whole molecule, peroxidase conjugate (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 18 hrs at 4°C followed by 1 hr at 22°C, and washed with several changes of PBS at 4°C. Sections were stained with the peroxidase chromagen 0.27% (w/v) diaminobenzidine (DAB) in Tris buffer, pH 7.6, plus 0.06% (v/v) H_2O_2 for 20 min at 22°C in the dark. This produces a brown dye in the vicinity of the bound antibody. Following a series of PBS rinses, the sections were fixed with formalin for 5 min and counterstained with hematoxylin.

For electron microscopy, frozen, thawed calfskin was minced into 1-mm^3 pieces and fixed with 0.05% glutaraldehyde and 3.0% paraformaldehyde in 0.1M phosphate buffer, pH 7.1, for 45 min at 22°C, and rinsed with several changes of phosphate buffer. Tissue blocks were dehydrated through a graded aqueous-ethanol series and infiltrated with and embedded in Lowicryl® K4M resin (Chemische Werke Lowi GmbH, Waldkraiburg, FRG). Sections, 60 nm thick, were cut and mounted on carbon-stabilized collodion-coated nickel grids. Sections on grids were pre-incubated on drops of Tris-buffered saline (TBS), pH 7.1, with 1.0% gelatin added, for 18 hrs at 4°C and rinsed with several changes of TBS, then incubated on drops of primary antibody (described above) for 3 hrs at 22°C, followed by several rinses with TBS. Control sections were incubated on drops of PBS without primary antibody. All sections were incubated with goat anti-rabbit IgG conjugated to 10 nm colloidal gold (AuroProbe® EM, Janssen Life Sciences Products, Olen, Belgium) for 3 hrs at 22°C, and subsequently rinsed with TBS plus 0.2% NaC1 followed by a brief rinse in distilled deionized water. Sections were stained with 5.0% aqueous uranyl acetate and lead citrate and were examined with a Zeiss EM-10B transmission electron microscope at 60 kV.

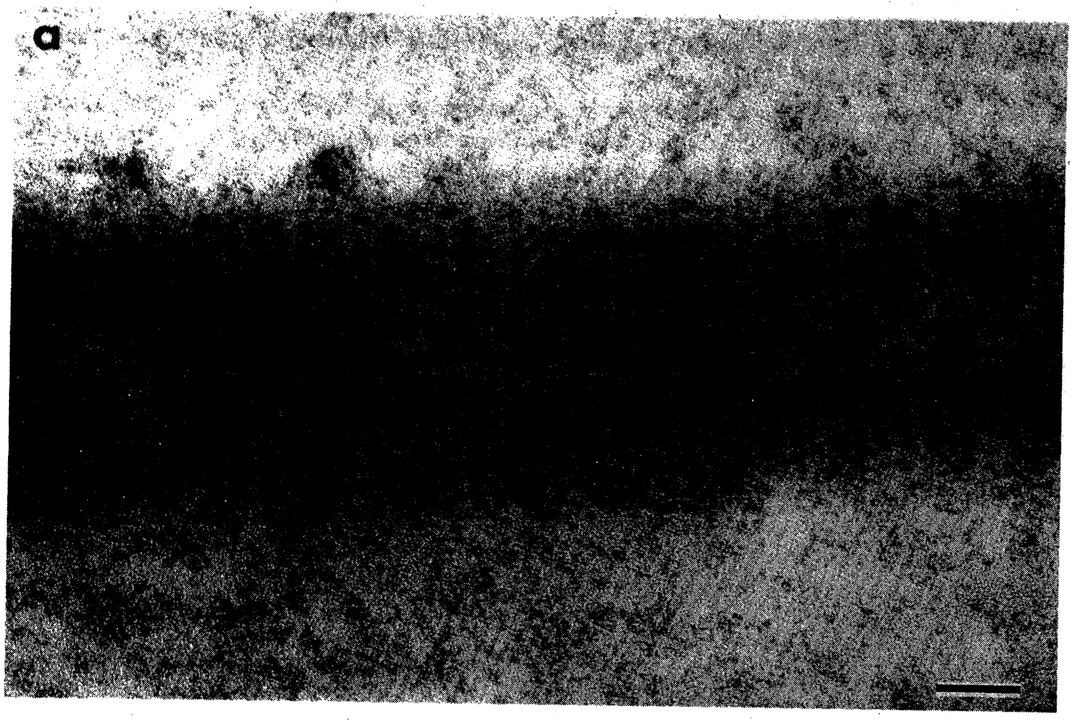
Type XII Collagen

Immunohistochemistry for type XII collagen was performed on skin dissected from 18-day chick embryos and stored frozen at −70°C. Frozen sections (6 μm thickness) for all experiments were collected on albumin-coated slides. To simulate liming, sections were treated with an aqueous calcium hydroxide solution for 17 hrs at4°C, then rinsed with a solution of PBS and 10 mM ethylenediaminetetraacetate. To simulate bating, sections were treated with a 0.1% solution of trypsin in PBS. To simulate pickling, tissue was incubated with a 0.22% H_2SO_4 solution with 1% added NaC1. All sections were then incubated with 0.15% hyaluronidase solution at 37°C for 30 min in a moist chamber and rinsed by PBS. All sections were then carried through sequential incubation steps with NGS, monoclonal anti-chicken collagen XII antibody, goat anti-mouse IgG conugated with peroxidase (Boehringer Mannheim Biochemicals), and the chromagen solution described above. Control slides were stained with hematoxylin following treatment with NGS. All other sections were dehydrated in a graded series of aqueous ethanol solutions, cleared in xylene, and mounted under a coverslip with permanent mounting medium.

Results

Fresh-frozen hide, following adsorption of antibody to type VI collagen, binds gold particles that have been treated so that they have an affinity for that antibody (q.v. Materials and Methods). These particles lie between adjacent fibrils but are not found on cut surfaces (Figures 1a). The interfibrillary gel in longitudinal sections (Figure 1b) and in cross sections (Figure 1c) also binds the type VI-labeling particles, as expected from previous observations that collagen type VI is found in the interfibrillary gel[5].

FIG. 1. — Electron micrographs of calf skin labeled with antibody-conjugated gold particles that bind selectively to antibody-collagen VI complexes. (a) Particles lying between two fibrils, but not on the cut surfaces; (b) particles in interfibrillary gel between longitudinal sections; (c) particles in interfibrillary gel among cross sections.



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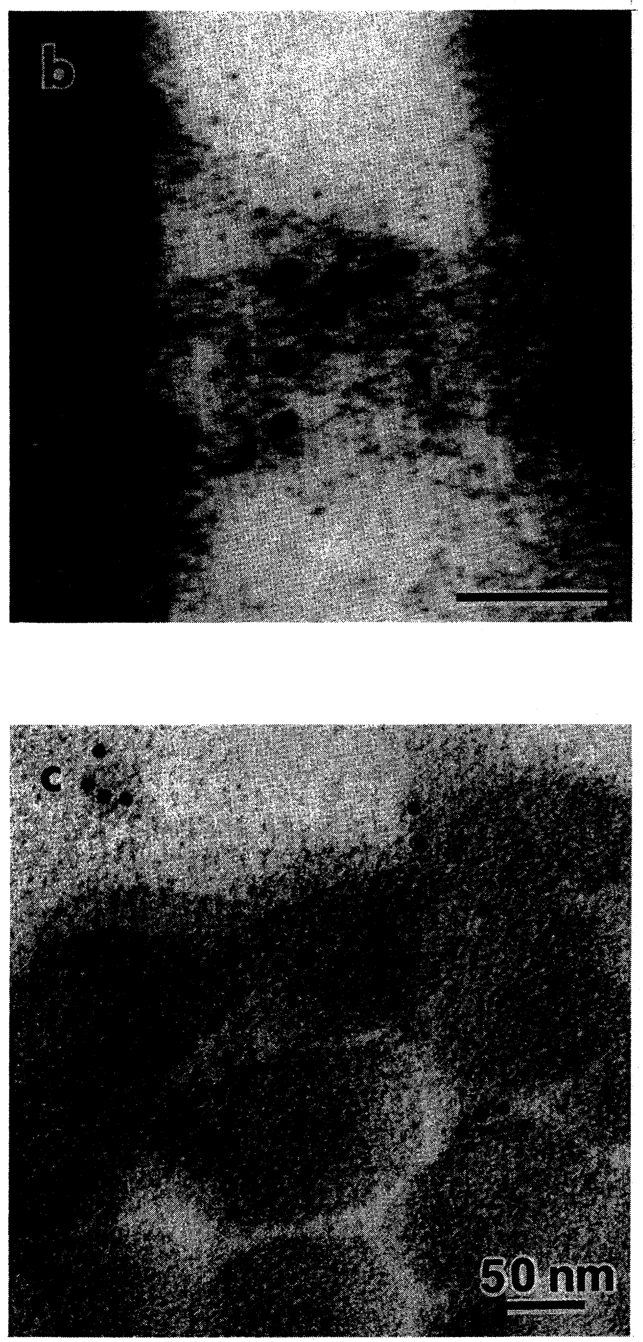


When stained with the brown dye associated with collagen VI (see Materials and Methods), the untreated calfskin viewed by light microscopy (Figure 2a) shows the minority collagen to be distributed throughout the corium, especially concentrated at the very top of the grain just under the unstained epidermis. Figure 2b shows untreated calfskin that was prepared with the same stain, but lacking the antibody.

FIG. 2. — Micrographs of calfskin (a) stained with chromogenic antibody to collagen type VI and (b) without antibody stain.

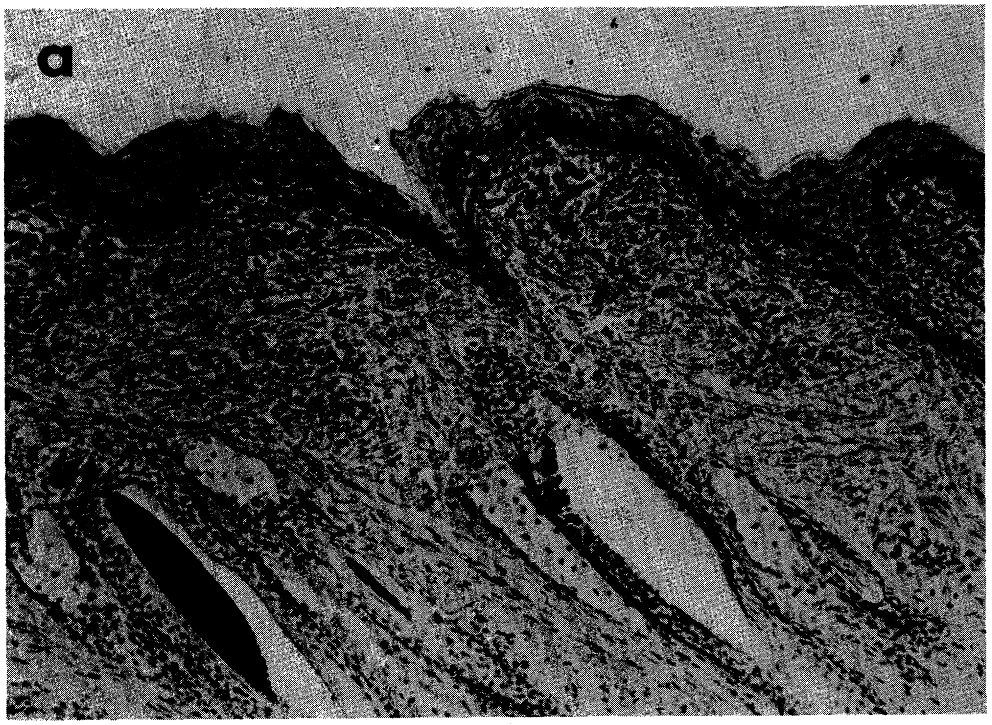
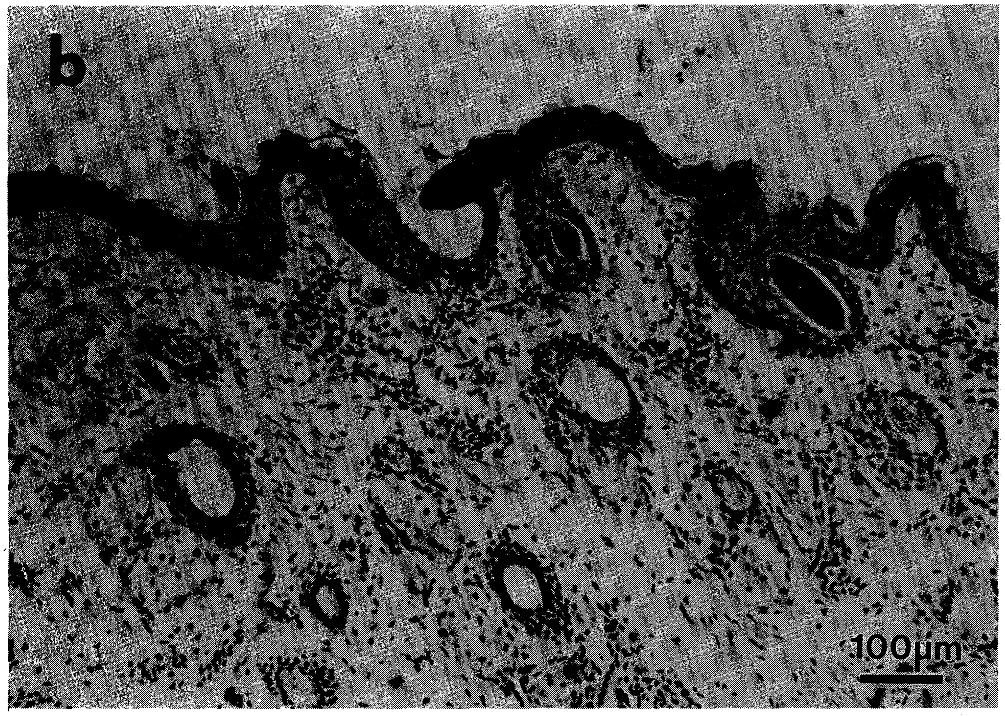


FIG. 2. — Micrographs of calfskin (a) stained with chromogenic antibody to collagen type VI and (b) without antibody stain.



After lime treatment, the structure of the corium and the epidermis is disrupted, but collagen VI persists with the same spatial distribution as before treatment (Figures 3a and b), and with no obvious reduction in amount. The collagen VI probably remains intact, since the antibody is sensitive to changes in molecular configuration. Collagen VI, therefore, persists through the lime. Indeed, as Figures 4 and 5 show, even sulfide and trypsin, which completely remove the hair and epidermis, fail to remove the collagen VI. The surface of the grain remains deeply stained.

FIG. 3. — Liming has no apparent effect on the presence of type VI collagen, as shown by persistence of chromogenic antibody to type VI collagen (dark areas). (a) calfskin grain; (b) calfskin corium.

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FIG. 4. — Persistence of type VI collagen after treatment with sulfide. Type VI collagen is stained with chromogenic antibody (dark areas).

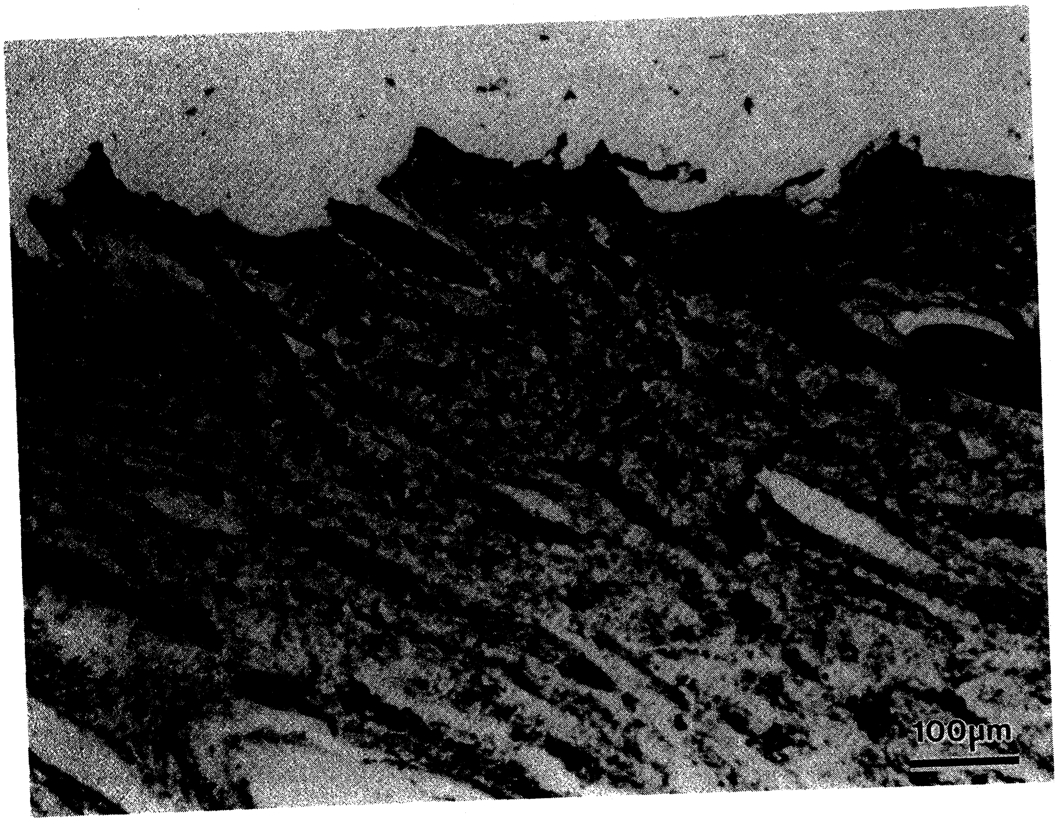
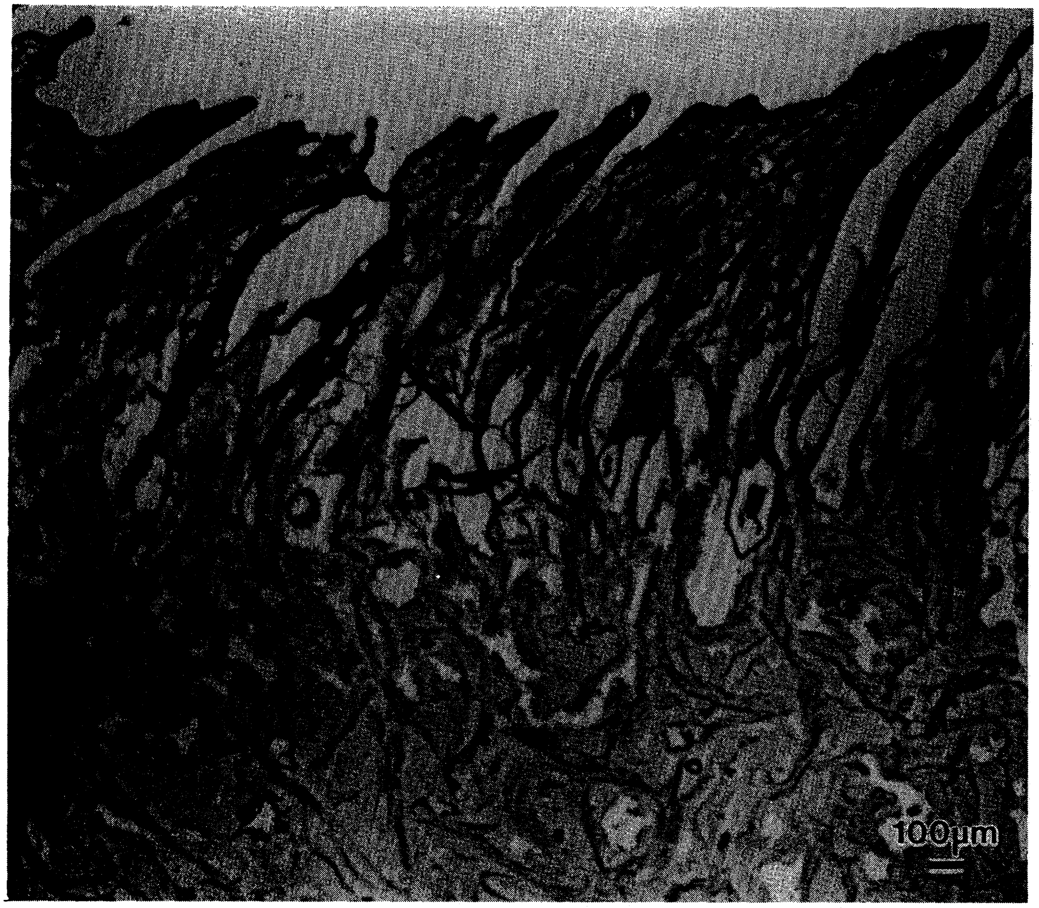
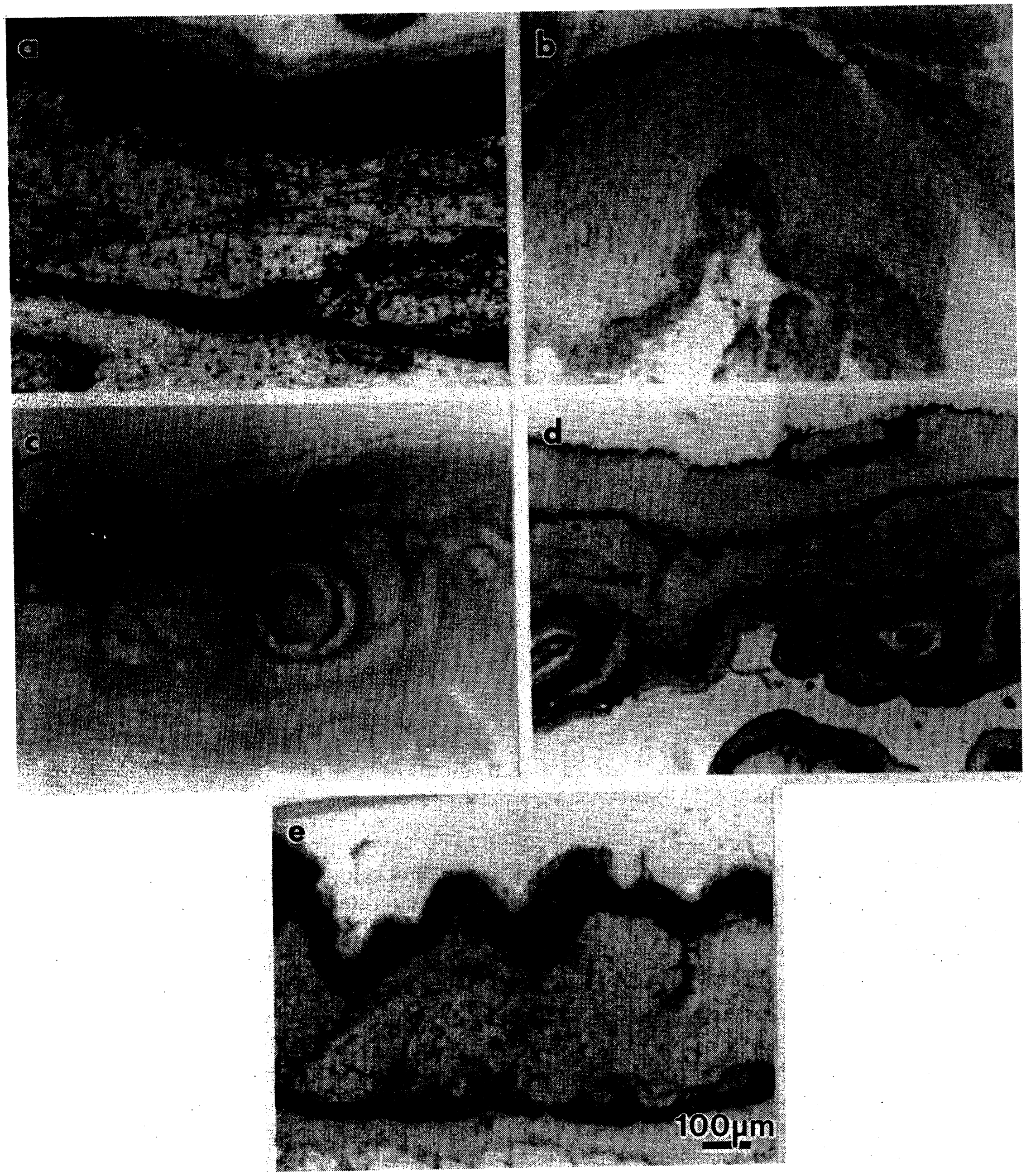


FIG. 5. — Persistence of type VI collagen after treatment with trypsin. Type VI collagen is stained with chromogenic antibody (dark areas).



To follow the fate of collagen type XII we used a monoclonal antibody to the variant of the collagen found in chick skin[9]. The untreated tissue, stained with hematoxylin and eosin but without antibody, is shown in Figure 6b. Figures 6a, c, d, e show the results of beamhouse chemistry applied to fetal chick skin. The details of the skin structure are not as clear in the fetal tissue as in the more mature calf skin shown in Figures 1-5, but the more-deeply stained areas, containing the FACIT collagen XII, had the birefringence of collagen fibers and so was associated with them. Figures 6a and d show that collagen XII persists through liming. Figure 6e shows that the acid treatment, used in pickling, also is ineffective in removing collagen XII, but the specimen shown in Figure 6c shows that trypsin, a typical enzyme used in bating, is sufficient by itself to remove it, or at least the part of the collagen molecule that binds to the monoclonal antibody.

FIG. 6. — Effects of beamhouse chemistry on collagen type XII in fetal chick skin. (a) untreated tissue stained with antibody (black areas); (b) Untreated tissue stained only with hematoxylin and eosin. Darker areas comprise collagen fibers; (c) trypsin-treated and stained with antibody; (d) lime-treated tissue stained with antibody; (e) pickled tissue stained with antibody.



Discussion

The chemistry that was applied to the specimens in this study was chosen to model treatment of hide in a beamhouse - liming, sulfide, bating, and pickling. The conditions of treatment were harsher than those of commercial processes. The specimens for light microscopy were at most 12 μm thick, permitting complete and instantaneous perfusion of the tissue with the reagents, with a minimum of pH buffering by the tissue. The bating enzyme was purer than usually expected in commercial bates, but the activity of the trypsin was high. All beamhouse reactions would have been expected to occur on the microscope slides.

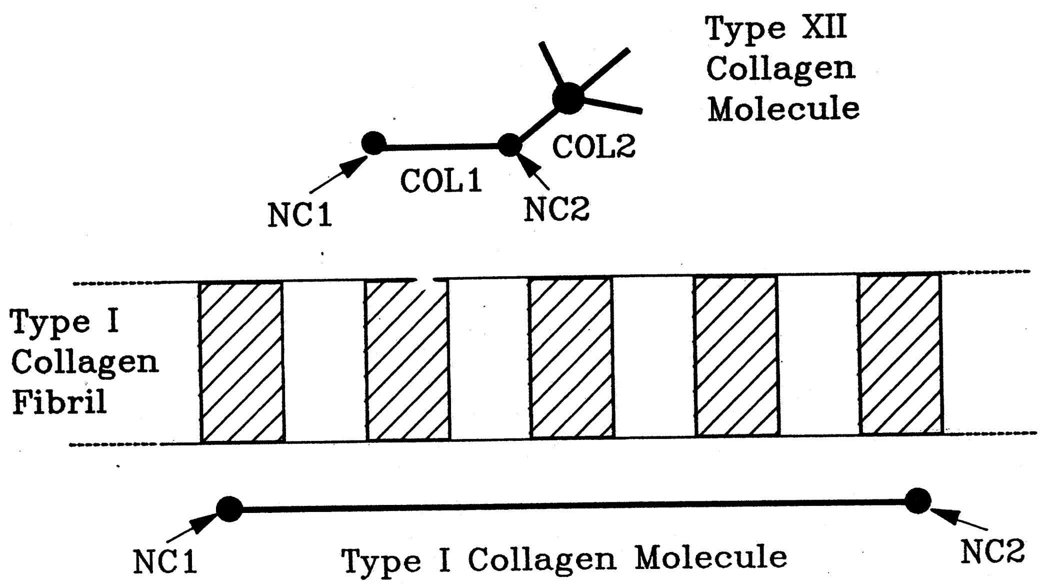
Collagen VI molecules, with their very large globular ends that prevent packing into fibrils[10], are very unlike the more familiar type I collagen. Their triple-helical domains, however, are chemically resistant, as are those of collagen I. It is expected that the globular domains are proteolyzed by trypsin. Even without the non-helical portions, which contain the domains that bind to collagen I and to cells[9], collagen VI remains through the beamhouse process.

It is not clear that this persistence should be called a failure of the process. Figure 3 showed that collagen VI is concentrated in a region that corresponds to the grain enamel. While in the corium this material might dry to a hard adhesive substance among the fibrils, which a leathermaker might want to remove, it might also be a component of a desirable layer that gives the surface of leather its rich quality. Collagen VII is also known to be localized at the surface of the grain[11].

Unlike the antibody used to study collagen VI, the antibody used in the collagen XII work was monoclonal, and could bind only to a non-triple-helical region at one end of the molecule, (NC-1 in Figure 7). Failure of the anti-collagen XII antibody to bind to the trypsin-treated specimen indicates that this portion, the recognized part of the molecule, had been removed, but other portions of the molecule, especially the triple-helical domains, might be present.

The molecule has been directly observed by electron microscopy in calfskin and has the peculiar shape depicted in Figure 7[1,9]. From indirect evidence[12] it is believed to lie along the surfaces of fibrils as drawn. It is in a critical location for influencing the proerties of leather after it is dried, since it would interact with fatliquor, dyes, and impregnants. If the non-collagenous domains were removed with trypsin, the remainder might be physically similar to the fiber-forming collagens in the rest of the fibril. The clustered charges that cause fribrillar packing, however, would be lacking. Thus, as with collagen VI, it is not completely clear what role these remnant collagen XII fragments would play in the properties of leather.

FIG. 7. — Domain structures of the molecules of collagen I and XII, and their supposed relation to the stained bands of the collagen fibril. All are drawn to the same scale.



The development of new reagents for syntans, fatliquors, and dyes depends on knowledge of the chemistry of the surface of fibrils. Continuing studies of fibril-surface modification by beaming and tanning will contribute to new developments.

Acknowledgments

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Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over other of a similar nature not mentioned.

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Discussion

MR. FRANK RUTLAND (Leather Industries Research Laboratory), Discussion Leader: Thank you for a very stimulating paper. The floor is now open for questions.

DR. K. J. BIENKIEWICZ (Leather Research Institute, Lodz, Poland): Dr. Kronick, I did not try to keep it a secret that there are several collagens in the hide. It was just a simplification when I spoke of "collagen" as such, because it was industrial collagen. Of course, we know that there are something like eight collagens in the hide, and there are other kinds in the cartilage, muscles and other organs. The collagen you have mentioned is probably accessible to the proteolytic enzymes. Are these collagens VI and XII attacked by proteolytic enzymes, which are not specific collagenases?

DR. KRONICK: It is clear that the non-collagenous regions are attacked, but the triple-helical portions are not.

DR. BIENKIEWICZ: So that is why they disappear during the beamhouse processes.

DR. KRONICK: If the helical regions were attacked, then collagen I would also be attacked, and you would have no hide left after bating.

DR. BIENKIEWICZ: Can you say what the contents are of collagen VI and XII?

DR. KRONICK: The content of collagen VI is perhaps .01% of the collagen I. It might be higher but I don't think it's lower.

DR. BINEKIEWICZ: So these are only of scientific interest at the moment?

DR. KRONICK: The point I was making is that the content is, of course, low, but the location is very strategic. One could have a lamella of these materials which could affect interfibrillar adhesion, and still have only a thousandth of the number of molecules of collagen type I, because the thickness of the fibrils contains the collagen type I which does not play a role in fibrillar adhesion.

DR. BILL PRENTISS (retired): I would like to pursue a little bit of what Dr. Bienkiewicz asked about the enzymes. You said "trypsin". I would like to know something about the nature of the trypsin you used in these experiments, because as you know, different proteolytic preparations have different effects on collagen, and I think tanners will tell you than even some conventional bating enzymes make holes in leather if the temperature gets out of control. It is therefore important to recognize that the preparation of trypsin that you use may be one of the reasons that you are seeing these effects. Some other preparation of trypsin or some other proteolytic enzyme might not show the same results.

DR. KRONICK: I think we have gone in the other direction. We have used highly-purified trypsin from a biochemical supply house, the best grade available, so that its purity is much better than that of the bating enzymes used by tanners. If this works or doesn't work, then bating enzymes should work or not work likewise.

DR. PRENTISS: I would suggest, Paul, that you might get one of your cohorts in the laboratory to run a bating test with this trypsin. If he were to measure the hydroxyproline released, and compare such release with that produced by our conventional bating enzymes then you would be sure.

DR. KRONICK: Okay, but I will say that the collagen I fibrils persist after the treatment in these experiments.

MR. BRUCE SHARP (Prime Tanning Company): Paul, you mention that collagen VI is unaffected in the presence of trypsin, and yet it was clear in your illustration that it seemed to be only the top layer of the grain that was left and you are calling this collagen VI. Is it possible that this material is this mysterious grain enamel which all tanners are trying to preserve?

DR. KRONICK: It is possible that it's a component of it.

MR. RUTLAND: Thank you Paul.

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